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(57) Abstract

The present invention relates to novel antibiotic peptides which possess antibacterial and/or antifungal activities causing no cytotoxicity, and to antibacterial and/or antifungal agents containing said peptides as active ingredients. In accordance with the present invention, it has been discovered that: a number of chemically-synthesized peptides which are derived from Tenecin, show superior antibacterial and/or antifungal activities, while causing no untoward effects, and they can be applied for the development of antibacterial and/or antifungal agents.

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NOVEL ANTIBIOTIC PEPTIDES

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to novel antibiotic peptides which possess superior antibacterial and/or antifungal activities while causing no cytotoxicity, and to antibacterial and/or antifungal agents containing said peptides as active ingredients.

Description of the Prior Art

More than 100 years have elapsed since the first scientific demonstration of microbial antagonism and five decades since the first clinical use of penicillin. At present, several thousand antibiotics are known and many of them are in practical use. However, studies on the antibiotics have been continuously needed due to the appearance of mutant microorganisms acquired resistance to the antibiotics and the serious side-effects of the commercially available antibiotics. In this regard, attempts to develop novel antibiotics to solve said problems have been carried out by screening secondary metabolites of microorganisms, by synthesizing analogues of known antibiotics such as quinolones or by isolating antibiotics such as proteins or peptides induced by an intracelluar defense mechanism(see: Natori S., J. Insect Physiol., 23:1169-1173(1977); Okada M. & Natori S., Biochem. J., 211:727-734(1983); Ando K. et al., Biochemistry, 260:7174-7177(1987); Steiner H. et al., Nature, 292:246-248(1981); Casteels, P. et al., Eur. J. Biochem., 187:381-386(1990)).

On the other hand, it has been known that insects protect themselves from pathogenic bacteria or parasites by their own cellular and humoral immune systems, and they

frequently respond to the attack of pathogens by producing antibiotics, e.g., antibacterial proteins or peptides. Until now, about 50 antibacterial proteins or peptides have been isolated from the insects and their structures have been also elucidated. Some of the self-protective proteins or peptides such as Cecropin, have been intensively studied, which provides basic ideas for the development of antibacterial substances whose modes of action are novel.

It has been also reported that most of antibacterial proteins or peptides may target lipid membrane, even though biological activities of all the antibacterial substances are not clearly understood. For example, Cecropin, which is appeared in the hemolymph of certain insects, shows its activity on Gram positive and negative bacteria, by the amphiphilic binding with lipid membrane of bacteria, to form ion channels diverse in size and to allow a rupture of cell membrane(see: Christensen, B. et al., Proc. Natl. Acad. Sci., USA, 85:5072-5076(1988)).

In addition to Cecropin, cysteine-containing Defensin and Sapecin, which are isolated from insects, are fallen within the antibacterial peptides whose target site are lipid membrane of Gram positive bacteria(see: Kuzuhara, T. et al., J. Biochem., 107:514-518(1990)). Their modes of action have been anticipated to be different from Cecropin, in light of the previous finding that insect Defensin leads to bacterial cell lysis in a relatively slower manner than Cecropin which requires only 1min to reach cell rupture.

Another antibacterial peptides whose target site are lipid membrane, includes Attacin, Sarcotoxin, Defterícin, Coleoptericin, Apidaecin and Abaecin. The peptides conserve G and P domains, and have an influence on the cell differentiation of Gram negative bacteria and, in turn, lead to chain-shaped cell growth. In particular, Attacin has been also reported to break down outer membrane of the targeted bacteria by inhibiting the synthesis of outer membrane proteins.

Besides the antibacterial peptides of insects

illustrated as above, several antibiotic peptides have been also isolated from amphibia, e.g., Magainin(see: Zasloff, M., Proc. Natl. Acad. Sci., USA, 84:5449-5453(1987), Ranalexin (see: Clark., D.P. et al., J. Biol. Chem., 269:10849-10855 (1994)), Brevinins(see: Morikawa, N. et al., Biochem. Biophys. Res. Commun., 189:184-190(1992)) and Esculantins (see: Simmaco, M. et al., FEBS Lett., 324:159-161(1993)). The peptides have been known to show their antibacterial activities in a similar mechanism to Cecropin, i.e., forming ion channels in lipid membrane of bacteria to rupture the cell.

On the other hand, the present inventors have previously reported the isolation of a protein which shows antibacterial activity(hereinafter referred to as "Tenecin") from a larva of Tenebrio molitor on which E. coli is infected(see: Lee, B.L. et al., J. Biochem., 116:53-58(1994)) whose amino acid sequences are:

VTCDILSVEAKGVKLNDAACAAHC-
LFRGRSGGYCNGKRVCVCR-CO_2H

However, the practical use of Tenecin have encountered several serious problems as followings: Tenecin should be isolated from the larva of Tenebrio molitor, which makes its isolation and mass production difficult; a large molecular size of Tenecin may provoke antigen-antibody reaction in vivo; Tenecin have narrow spectrum of target cell, i.e., on Gram positive bacteria only; and, Tenecin is unstable since its chemical nature is protein.

Under the circumstances, in order to provide novel antibacterial substances to overcome the problems of Tenecin discussed above, the present inventors have synthesized peptide fragments of Tenecin and their chemical analogues by the addition, deletion or substitution of amino acids, based on a finding that active site of Tenecin is conserved on a specific region, and finally discovered that the peptides possess superior antibacterial and

antifungal activities while causing no untoward effects such as cell lysis.

SUMMARY OF THE INVENTION

In accordance with the present invention, the inventors discovered that: a number of chemically-synthesized peptides which are derived from Tenecin, show superior antibacterial and/or antifungal activities, while causing no untoward effects, and they can be applied for the development of antibacterial and/or antifungal agents.

A primary object of the present invention is, therefore, to provide novel antibiotic peptides which possess superior antibacterial and/or antifungal activities while causing no cytotoxicity.

The other object of the invention is to provide antibacterial and/or antifungal agents containing said peptides as active ingredients.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides acid- or amide-form peptides which possess both antibacterial and antifungal activities, which are represented by the general formula(I), formula(II) and formula(III) as below, and analogues thereofs including enantiomers, retro-inversoes and derivatives where at most 3 neighboring amino acids located in each of N- and/or C-terminals, or at most 2 neighboring amino acids in the mid-part of the peptides, are substituted with D-form amino acids, respectively:

$$\alpha^1\ \alpha^2\ \alpha^3\ \alpha^4\ \alpha^5\ V\ C\ V\ C\ \alpha^6 \qquad (I)$$

wherein,

α^1 is 2 to 4 residues of amino acid, which are

preferably selected from the group consisting of YC, FC, WS, FYC, KYC, PYC, KFYC and FFYC;

α^2 is N, K or V;

α^3 is vacant, or G, P, L or K;

α^4 is vacant or K;

α^5 is vacant or R;

α^6 is R, L or D; and,

C may be replaced with aminoisobutyric acid.

$$\beta^1\ \beta^2\ \beta^3\ \beta^4\ \beta^5\ \beta^6\ \beta^7\ \beta^8\ \beta^9\ \beta^{10} \qquad \text{(II)}$$

wherein,

β^1 is vacant, or 1 or 2 basic amino acids;

β^2 is vacant, or 1 or 2 hydrophobic or basic amino acids(provided that β^1 is vacant and β^2 is 1 residue of amino acid, Pro and Tyr are excluded);

β^3 is 2 amino acids selected from the group consisting of hydrophobic amino acids and Cys;

β^4 is 1 or 2 amino acids(provided that β^4 is 1 residue of amino acid, Pro and acidic amino acids are excluded; and, provided that β^4 is 2 amino acids, both of which should not be acidic amino acids);

β^5 is 1 or 2 basic amino acids;

β^6 is vacant, or a hydrophobic amino acid;

β^7 is an amino acid selected from the group consisting of hydrophobic aromatic and aliphatic amino acids, Cys and Ser;

β^8 is a hydrophobic amino acid;

β^9 is an amino acid selected from the group consisting of hydrophobic aromatic and aliphatic amino acids, Cys and Ser (provided that β^7 is a hydrophobic

aliphatic amino acid or Ser, β^9 should be a hydrophobic aromatic amino acid or Cys); and,

β^{10} is 1 or 2 basic amino acids.

$$\gamma^1\ \gamma^2\ \gamma^3\ \gamma^4\ \gamma^5 \qquad \text{(III)}$$

wherein,

γ^1 is 1 to 4 residues of amino acid;
γ^2 is 2 to 4 hydrophobic amino acids;
γ^3 is 1 or 2 basic amino acids;
γ^4 is 2 to 4 hydrophobic amino acids; and,
γ^5 is 1 to 3 amino acids containing at least one of basic amino acids(provided that γ^5 is more than 2 amino acids, basic amino acids are directed to N-terminal).

The present invention also provides acid- or amide-form peptides which possess antifungal activity, which are represented by the general formula(IV) as below, and analogues thereofs including enantiomers, retro-inversoes and derivatives where at most 3 neighboring amino acids located in each of N- and/or C-terminals, or at most 2 neighboring amino acids in the mid-part of the peptides, are substituted with D-form amino acids, respectively:

$$\delta^1\ (ab)_{n1}\ (ba)_{n2}\ c\ \delta^2 \qquad \text{(IV)}$$

wherein,

δ^1 is 1 to 4 residues of amino acid;
a is a hydrophobic aromatic amino acid;
b is a hydrophobic aliphatic amino acid;
n1 is an integer of 1 or 2;
n2 is an integer of 1, 2 or 3(provided that n1 is 1, n2 is 2 or 3; and, provided

that n1 is 2, n2 is 1 or 2);
c is vacant, or a hydrophobic amino acid;
and,
δ^2 is 1 or 2 basic amino acids.

In describing the antibiotic peptides of the present invention, one-letter abbreviation of amino acids is employed, in accordance with the nomenclature system of the IUPAC-IUB.

Further, the term basic amino acids are employed to mean usual or unusual amino acids with a basic side chain, e.g., His, Lys & Arg as an usual and 2-methyl-L-arginine, ornithine, 2,3-diaminopropionic acid and 2,4-diaminobutyric acid as an unusal.

Hydrophobic amino acids are employed to mean both aromatic and aliphatic amino acids, where the hydrophobic aromatic amino acids contain usual or unusual amino acids with an aromatic functional group, e.g., Phe, Tyr, Trp & Pro as an usual and L-3-(2,5-dihydrophenyl)-alanine, L-β-(5-hydroxy-2-pyridyl)-alanine and β-isotyrosine as an unusual; and, the hydrophobic aliphatic amino acids contain usual or unusual amino acids with an aliphatic functional group, e.g., Gly, Ala, Val, Leu & Ile as an usual and aminoisobutyric acid, isovaline, norleucine, norvaline, 2-amino-5-methylhexanoic acid, 2-amino-6-methylheptanoic acid, 2-amino-7-methyloctanoic acid as an unusual.

Acidic amino acids are employed to mean usual or unusual amino acids with an acidic functional group, e.g., Asp & Glu as an usual.

Usual amino acids are employed to mean naturally occuring 20 amino acids named in conformity with the IUPAC-IUB nomenclature system, and unusual amino acids are all the amino acids except for said naturally occurring 20 usual amino acids.

Though peptides are synthesized chemically in the

present invention, they can also be prepared from the host cells transformed with proper recombinant plasmids containing the nucleotide sequences which are reversely deduced from the amino acid sequences of peptides of interest.

Based on the determination of minimal inhibition concentrations(MICs) against test organisms, i.e., bacteria and fungi, it has been found that peptides of the invention possess excellent antibacterial and/or antifungal activities. Further, from the absorbance measurement after coincubation of red blood cells and the peptides, it has been also found that the peptides do not give rise to lyse the red blood cells.

From the above results, it has been concluded that: the antibiotic peptides of the invention can be applied for the development of antibacterial and antifungal agents for the chemotherapy of local and systemic infections caused by pathogenic bacteria and/or fungi; and, they can be formulated into potent antibacterial and/or antifungal agents with pharmaceutically acceptable carriers.

For oral administration, the peptides can be formulated into a solid preparation such as tablets, pills, granules, powder, capsules and the like, or a liquid preparation such as solutions, suspensions, emulsions and the like. The pharmaceutical preparations for oral administration can contain active peptide or peptides alongside the customary excipients such as (a) fillers and extenders, for example starches, lactose, sucrose, glucose, mannitol and silica, (b) binders, for example carboxymethylcellulose, alginates, gelatine and polyvinylpyrrolidone, (c) humectants, for example glycerine, (d) disintegrating agents, for example agar-agar, calcium carbonate and sodium carbonate, (e) solution retarders, for example paraffin, (f) absorption accelerators, for example quaternary ammonium compound, (g) wetting agents, for example cetyl alcohol or glycerine

monostearate, (h) adsorbents, for example kaolin and bentonite, (i) lubricants, for example talc, calcium stearate and magnesium stearate and solid polyethylene glycols, (j) colorants, (k) flavourings, (l) sweeteners, or mixtures of the substances listed under (a) to (l).

When the preparation is used for parental administration, the preparation is made in an injection formula, an intravenous drip infusion and the like. For the preparation of an injection formula, the solutions and emulsions can be in a sterile form which is isotonic with blood. The suspensions can contain in addition to the active peptide or peptides, preservatives, stabilizers, solubilisers, wetting agents, salts for changing the osmotic pressure or buffers.

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

Example 1: Chemical synthesis of peptide fragments of Tenecin and determination of active site

In order to determine the locus of active site, a number of peptide fragments of Tenecin were chemically synthesized from N-terminal to C-terminal, in accordance with the solid phase synthesis method:

The peptides were synthesized by employing a peptide synthesizer(Applied Biosystem Instrument, Model 431A, USA). For the chemical synthesis, free amino acids were coupled to Fmoc(9-fluorenylmethoxycarbonyl) group for N-terminal protection, and Trt(trityl), Boc(butyloxycarbonyl), tBu(t-butylester) or Pmc(pentamethylchroloman) group for protection of reactive side chains. Amino acids thus protected includes: Fmoc-L-Ala, Fmoc-L-Arg(Pcm), Fmoc-L-Asn(Trt), Fmoc-L-Asp(tBu), Fmoc-L-Cys(Trt), Fmoc-L-Gly, Fmoc-L-Glu(tBu), Fmoc-L-Gln(Trt), Fmoc-L-His(Trt), Fmoc-L-Ile, Fmoc-L-Leu, Fmoc-L-Lys(Boc), Fmoc-L-Phe, Fmoc-L-Ser,

Fmoc-L-Thr(tBu) and Fmoc-L-Val.

In order to increase the stability of synthesized peptides, PAL(5-(4-amino)methyl-3,5-dimethoxyphenoxy-valeric acid) and WANG(4-alkoxybenzyl alcohol) resins were employed, to give peptides having amide- and acid-forms of C-terminal upon cleavage from the resins, respectively, where 0.1mmole of the resin and 0.5mmole of amino acids were preferably added.

HOBt(N-hydroxybenzotriazole) and DCC(N,N'-dicyclohexyl carbodiimide) were employed as a carboxyl group activator in coupling of amino acids. After the completion of coupling for about 35min, Fmoc N-terminal protecting group was removed by the treatment of piperidine. On the other hand, the side chain protected peptide on the solid support resin, was reacted with a cleavage solution(containing 80% TFA(trifluoroacetic acid), 2.5% ethanedithiol, 5% thioanizole, 7.5% phenol and 5% H_2O) for 8hrs at room temperature, and then, the peptide from which side chain protecting group was removed, was isolated from the resin. A solution containing the synthesized peptide was obtained by filtration and TFA was removed by purging nitrogen gas. To the resultant, diethylether chilled at -20°C was added and then centrifuged at 3,000rpm for 20min to precipitate the peptide.

Peptides thus prepared were purified with the aid of preparative HPLC equipped with reverse phase C_{18} column (Delta Pak C18-300A,, 1.9 x 30cm, Waters, USA) by eluting with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 20mL/min, and their molecular weights were determined by mass spectroscopy.

Antibacterial and antifungal activities of the peptide fragments of Tenecin thus synthesized, were determined by MIC tests employing test organisms, i.e., Staphylococcus aureus and Candida albicans, as fully described in Example 2 below. Amino acid sequences of the peptides and their antibacterial and antifungal activities were disclosed in

Table 1.

Table 1: Peptide fragments of Tenecin and antibiotic activity thereofs

Name	Amino acid sequence	Location in Tenecin	MIC(μg/ml)	
			S. aureus	C. albicans
TEA	DAACAAHHCLFR-NH_2	middle	>500	>100
TEB	NDAACAAHCLFRGRSGG-NH_2	meddle	>500	>100
TEC	VTCDILSVEAKGVKL-NH_2	N-terminal	>500	>100
TED	YCNGKRVCVCR-NH_2	C-terminal	10	10
TEO	LSVEAKGVKLNDAACAAHCL-NH_2	middle	>500	>100
TEQ	LSVEAKGVKLGGGYCNGKRVCVCR-NH_2	middle and C-terminal	>500	>100
Tenecin	VTCDILSVEAKGVKLNDAACAAHCLFRGR-SGGYCNGKRVCVCR-CO_2H		3	>500

As can be seen in Table 1, it was surprisingly determined that TED possesses an excellent antifungal activity which is not appeared in Tenecin, though all of the peptide fragments showed lower antibacterial activity than that of intact Tenecin.

To improve antibacterial and antifungal activities of the peptides, based on the results above, amino acids were added to N-terminal of the TED peptide: for example, TEDF was prepared by adding Phe to N-terminal of the TED peptide, and TEDFK was prepared by adding Phe and Lys to N-terminal. TED, TEDF and TEDFK thus prepared further underwent a multiplicity of addition, deletion and replacement of amino acids in the following examples.

Example 2: Determination of antibacterial and antifungal activities

Antibacterial and antifungal activities of the peptides were determined by employing MIC test as follows:

To determine antifungal activity, a test organism Candida albicans(ATCC 36232) was cultured on Sabouraud dextrose agar plate for 24 to 48hrs, and colonies thus grown were suspended on Sabouraud dextrose medium(Gibco,

USA) to have $O.D._{530}$ = 0.5(3 x 10^6 cells) and further diluted with 100-fold to have $O.D._{530}$ = 0.005(3 x 10^4 cells). 100μl of Candida albicans culture thus prepared was pipetted on 96-well microplates which had already contained 100μl of a serially diluted peptide solution. After incubation at 30°C for 24hrs, absorbance at 530nm was observed to determine the MIC of peptide against Candida albicans.

Antibacterial activity of peptide was determined analogously as above, with an exception that Staphylococcus aureus(ATCC 6538) selected as a test organism was cultured on M-3 medium(Gibco, USA) at 37°C and cell number was adjusted to 2 x 10^8 cells before 100-fold dilution.

A number of peptides whose antibacterial and antifungal activities were determined as aboves, were shown in Tables 2, 3, 4, 5 and 6.

Table 2: Modification of N-terminal amino acid residues of the TED peptide

Name	Amino acid sequence	MIC(μg/ml) against C. albicans
TED	YCNGKRVCVCR-NH_2	10
TED11	GKRVCVCR-NH_2	>500
TED12	KGKRVCVCR-NH_2	>500
TED13	NGKRVCVCR-NH_2	>500
TED14	SNGKRVCVCR-NH_2	30 to 50
TED15	CNGKRVCVCR-NH_2	>500
TED16	FCNGKRVCVCR-NH_2	10 to 20
TED17	LCNGKRVCVCR-NH_2	100
TED18	KCNGKRVCVCR-NH_2	>500
TED19	WSNGKRVCVCR-NH_2	10 to 20

Table 3: Addition of an amino acid to N-terminal of the TED peptide

Name	Amino acid sequence	MIC(μg/ml)	
		S. aureus	C. albicans
TED	YCNGKRVCVCR-NH_2	10	10
TEDF	FYCNGKRVCVCR-NH_2	5	10
TEDK	KYCNGKRVCVCR-NH_2	5	5
TEDP	PYCNGKRVCVCR-NH_2	5	5
TEDS	SYCNGKRVCVCR-NH_2	10	50
TEDA	AYCNGKRVCVCR-NH_2	10	10
TEDD	DYCNGKRVCVCR-NH_2	10	50
TEDL	LYCNGKRVCVCR-NH_2	5	5
TEDW	WYCNGKRVCVCR-NH_2	20	20

Table 4: Addition of an amino acid to N-terminal of the TEDF peptide

Name	Amino acid sequence	MIC(μg/ml)	
		S. aureus	C. albicans
TEDF	FYCNGKRVCVCR-NH_2	5	10
TEDFK	KFYCNGKRVCVCR-NH_2	5	3
TEDFF	FFYCNGKRVCVCR-NH_2	5	3
TEDFP	PFYCNGKRVCVCR-NH_2	>500	>500
TEDFS	SFYCNGKRVCVCR-NH_2	50	10
TEDFY	YFYCNGKRVCVCR-NH_2	50	100
TEDFD	DFYCNGKRVCVCR-NH_2	>500	>500
TEDFL	LFYCNGKRVCVCR-NH_2	30	100
TEDFW	WFYCNGKRVCVCR-NH_2	1	10

Table 5: Modification of the TEDFK peptide

Name	Amino acid sequence	MIC(μg/ml) against C. albicans
TEDFK	KFYCNGKRVCVCR-NH_2	3
TEDFK-1	KFYCNKRVCVCR-NH_2	5
TEDFK-2	KFYCNGRVCVCR-NH_2	5
TEDFK-3	KFYCNPKRVCVCR-NH_2	5
TEDFK-4	KFYCNLKRVCVCR-NH_2	5
TEDFK-5	KFYCNKKRVCVCR-NH_2	5
TEDFK-6	KFYCVGKRVCVCR-NH_2	5
TEDFK-7	KFYCKGKRVCVCR-NH_2	3
TEDFK-8	KFYCNGKVCVCR-NH_2	5
TEDFK-9	KFYCNPGPVCVCR-NH_2	20
TEDFK-10	KFYCNGKRVCVCL-NH_2	5
TEDFK-11	KFYCNGKRVCVCD-NH_2	5

Table 6: Substitution of Cys in the TEDFK peptide with aminoisobutyric acid(Aib)

Name	Amino acid sequence	MIC(μg/ml) against C.albicans
TEDFK	KFYCNGKRVCVCR-NH_2	3
TEDFK-1A	KFY(Aib)NGKRVCVCR-NH_2	1 to 3
TEDFK-2A	KFYCNGKRV(Aib)VCR-NH_2	5
TEDFK-3A	KFYCNGKRVCV(Aib)R-NH_2	20
TEDFK-1,2A	KFY(Aib)NGKRV(Aib)VCR-NH_2	20

As can be seen in Tables 2 to 6, it was clearly demonstrated that peptide TED, TEDF, TEDFK and derivatives thereofs of the present invention possess excellent antibacterial and/or antifungal activities.

Example 3: Comparison of antifungal activity of the synthesized peptides and Ketoconazole

Derivatives of peptide TEDFK whose amino acid sequences are disclosed in Tables 5 and 6, were subjected to a comparison of the antifungal activity with a commercially available antifungal drug, Ketoconazole (Janssen Foundation, Belgium).

Table 7: Antifungal activity of the synthesized peptides and Ketoconazole

Name	MIC(μg/ml) against C.albicans
Ketoconazole	6
TEDFK-2	6
TEDFK-3	6
TEDFK-5	3
TEDFK-6	6
TEDFK-7	3
TEDFK-8	6
TEDFK-9	12
TEDFK-10	6
TEDFK-11	6
TEDFK-1A	2
TEDFK-2A	6
TEDFK-3A	12

As can be seen in Table 7, it was determined that some of TEDFK peptide derivatives exhibit much higher antifungal activity than that of Ketoconazole.

Example 4: Antibiotic activity of TEDFK peptide analogues(I)

Further modifications of a peptide TEDFK were made by employing addition, deletion and substitution of amino acids, to prepare a number of peptide derivatives which can be classified as 3 groups fallen within the general formula(II), (III) and (IV), respectively. Antibacterial and antifungal activities for the peptide derivatives were determined analogously as in Example 2(see: Tables 8, 9 and 10).

Table 8: Antibiotic activity of TEDFK peptide derivatives of group 1

Name	Amino acid sequence	MIC(μg/ml)	
		S. aureus	C. albicans
TEDFK	KFYCNGKRVCVCR-NH_2	6.25	6.25
M1	KKYCNGKRVCVCR-NH_2	3.16	3.12
M2	KPYCNGKRVCVCR-NH_2	6.35	6.25
M3	FKYCNGKRVCVCR-NH_2	12.7	12.5
M4	KKYCKKRVCVCR-NH_2	3.16	3.12
M5	KKYCNKKCVCK-NH_2	3.16	3.12
M6	KKYCNKKCVCK-NH_2	3.16	3.12
M8	KFY(Aib)KKVCVCK-NH_2	3.16	3.12
M9	KFYINGKRVCVCR-NH_2	3.16	3.12
M10	KFYSNGKRVCVCR-NH_2	6.35	6.25
M11	KFYCNGKRVSVCR-NH_2	6.35	6.25
M12	KFYCNGKRVCVnLR-NH_2	6.35	6.25
M13	KFYCNGKRICICR-NH_2	6.35	6.25
M14	KFY(Aib)NGKRVIVCR-NH_2	12.7	12.5
M15	YCNGKRVCVCRKK-NH_2	6.35	6.25
M16	KYCNGKRVCVCRK-NH_2	6.35	6.25
M17	KFY(Aib)KGKRVCVCR-NH_2	6.35	6.25
M18	KFYCDGKRVCVCR-NH_2	12.7	12.5
M19	KFY(Aib)NGKKVFVFK-NH_2	6.35	6.25
M21	KIIINKKICICK-NH_2	3.16	3.12
M25	KFYCNGKRV(Aib)VCK-NH_2	6.35	6.25
M26	KWYCNGKRVCVCR-NH_2	6.35	6.25
M27	KFYCNGKRVWVCR-NH_2	6.35	6.25
M28	KFYCNGKRVCVWR-NH_2	12.7	12.5
M29	WFYCNGKRVCVCR-NH_2	30	25
M30	KFYCNWKRVCVCR-NH_2	12.7	12.5
M31	KFYCNGKRVCVCW-NH_2	12.7	12.5

* nL: norleucine, Aib: aminoisobutyric acid

Table 9: Antibiotic activity of TEDFK peptide derivatives of group 2

Name	Amino acid sequence	MIC(μg/ml)	
		S. aureus	C. albicans
TEDFK	KFYCNGKRVCVCR-NH_2	6.25	6.25
M20	KFY(Aib)KKVFVFK-NH_2	6.35	6.25
M22	KKYIKKVFVFK-NH_2	3.16	3.12
M23	KYIKKVFVFK-NH_2	3.16	3.12
M24	KKKYIKKVFVFK-NH_2	3.16	3.12
M35	KKYIKKYIKK-NH_2	12.7	12.5
M36	KVFVFKFVFVK-NH_2	6.25	6.25

* Aib: aminoisobutyric acid

As can be seen in Tables 8 and 9, it was clearly demonstrated that TEDFK peptide derivatives of group 1 and 2 show both antibacterial and antifungal activities.

Table 10: Antifungal activity of TEDFK peptide derivatives of group 3

Name	Amino acid sequence	MIC(μg/ml) against C. albicans
TEDFK	KFYCNGKRVCVCR-NH_2	6.25
M32	KKKKYIVFVFK-NH_2	3.12
M33	KKKYIVFVFK-NH_2	3.12
M34	KKYIVFVFK-NH_2	3.12
M37	KYIVFVFK-NH_2	3.12

As can be seen in Table 10, it was determined that TEDFK peptide derivatives of group 3 possess excellent antifungal activity.

Example 5: Antibiotic activity of TEDFK peptide analogues(II)

Enantiomers and retro-inversoes of TEDFK were prepared and their antibiotic activities were determined analogously as in Example 2. In addition, derivatives of M22 peptide shown in Table 9 where at most 3 neighboring amino acids

located in each of N- and/or C-terminals, or at most 2 neighboring amino acids in the mid-part of the peptide, were substituted with D-form amino acids, were prepared, and their antibacterial and antifungal activities were also determined(see: Table 11).

Table 11: Antibiotic activity of TEDFK peptide derivatives(*)

Name	Amino acid sequence	MIC(μg/ml)	
		S. aureus	C. albicans
TEDFK	KFYCNGKRVCVCR-NH_2	6.25	6.25
Enantiomer of TEDFK	kfycngkrvcvcr-NH_2	6.35	6.35
Retro-inverso of TEDFK	rcvcvrkgncyfk-NH_2	6.25	6.25
M22	KKYIKKVFVFK-NH_2	3.16	3.12
M22-1	kKYIKKVFVFK-NH_2	3.12	3.10
M22-2	kkYIKKVFVFK-NH_2	3.15	3.12
M22-3	kkyIKKVFVFK-NH_2	3.16	3.12
M22-4	KKYIKKVFVFk-NH_2	3.17	3.11
M22-5	KKYIKKVFVfk-NH_2	3.12	3.12
M22-6	KKYIKKVFvfk-NH_2	3.13	3.15
M22-7	kKYIKKVFVFk-NH_2	3.11	3.12
M22-8	kkYIKKVFVFk-NH_2	3.16	3.12
M22-9	kkyIKKVFVFk-NH_2	3.15	3.11
M22-10	kKYIKKVFVfk-NH_2	3.17	3.15
M22-11	kkYIKKVFVfk-NH_2	3.16	3.14
M22-12	kkyIKKVFVfk-NH_2	3.15	3.15
M22-13	kKYIKKVFvfk-NH_2	3.18	3.15
M22-14	kkYIKKVFvfk-NH_2	3.13	3.11
M22-15	kkyIKKVFvfk-NH_2	3.12	3.12
M22-16	KkYIKKVFVFK-NH_2	3.15	3.16
M22-17	KKyIKKVFVFK-NH_2	3.15	3.12
M22-18	KKYiKKVFVFK-NH_2	3.16	3.15
M22-19	KKYIkKVFVFK-NH_2	3.15	3.14
M22-20	KKYIKkVFVFK-NH_2	3.16	3.12
M22-21	KKYIKKvFVFK-NH_2	3.17	3.15

Table 11: (continued)

M22-22	KKYIKKVfVFK-NH_2	3.15	3.12
M22-23	KKYIKKVFvFK-NH_2	3.16	3.17
M22-24	KKYIKKVFVfK-NH_2	3.14	3.11
M22-25	KkyIKKVFVFK-NH_2	3.15	3.12
M22-26	KKyiKKVFVFK-NH_2	3.16	3.15
M22-27	KKYikKVFVFK-NH_2	3.17	3.11
M22-28	KkYIkkVFVFK-NH_2	3.15	3.12
M22-29	KKTIKkvFVFK-NH_2	3.15	3.11
M22-30	KKYIKKvfVFK-NH_2	3.17	3.12
M22-31	KKYIKKVfvFK-NH_2	3.16	3.15
M22-32	KKYIKKVFvfK-NH_2	3.15	3.12

*: small letters represent D-form amino acids.

As shown in Table 11, it was proved that enantiomers and retro-inversoes of TEDFK, and derivatives of M22 peptide possess both antibacterial and antifungal activities to the level of TEDFK and M22 peptide, respectively.

From the above results, it was determined that the analogues, i.e., enantiomers, retro-inversoes and derivatives where at most 3 neighboring amino acids located in each of N- and/or C-terminals, or at most 2 neighboring amino acids in the mid-part of the peptides, are substituted with D-form amino acids, have similar activities to those of the parent peptides of the present invention, which is correlated with the previous reports that antibiotic peptides have no stereo-specificity for the targeted membrane since the peptides interact the membrane without specific binding with chiral receptor or enzyme (see: Bessalle, R. et al., FEBS Lett., 274:151-155(1990); Wade, D. et al., Proc. Natl. Acad. Sci., USA, 87:4761-4765(1990); Matsuzaki, K. et al., Biochemistry, 34:3423-3429(1995); Merrifield, R.B. et al., Proc. Natl. Acad. Sci., USA, 92:3449-3453(1995); Krause, E. et al., Anal. Chem., 67:252-258(1995)).

Example 6: Cytotoxicity

To examine whether the peptides synthesized in the invention cause cell lysis or not, red blood cells were first obtained by the centrifugation of 3ml of human blood, rinsed with PBS(phosphate buffered saline) solution three times and diluted with the same solution to have 20ml in total. To 190μl of the red blood cell-containing solution thus prepared, was added 10μl of peptide solution prior to the incubation at 37°C for 30min. After incubation, centrifugation followed to obtain supernatant. Then, a level(%) of cell lysis caused by peptides was determined by the examination of absorbance at 576nm(see: Table 12). At this moment, TEDFK, TEDFK-1A, M5, M6, M19, M22 and M32 were chosen as peptide fragments of Tenecin, and an antibiotic peptide KLK(see: Natori, S. et al., J. Biochem., 117(6):1312-1316(1995)) and a commercially available antibiotic, Amphotericin B(Sigma, USA) known to lyse a red blood cell were employed as controls, respectively.

Table 12: Lysis of red blood cell(%)

Peptide concentration (μg/ml)	Cell lysis(%)								
	TEDFK	TEDFK-1A	M5	M6	M19	M22	M32	KLK*	Amphotericin B
0.078	0	0	0	0	0	0	0	0	0
0.625	0	0	0	0	0	0	0	0	8.06
7.8	0	0	0	0	0	0	0	0	100
31.2	0	0	0	0	0	0	0	9.6	100
62.5	0	0	0	0	0	0	0	78.6	100
125	0	0	0	0	0	0	0	89.9	100
250	0	0	0	0	0	0	0	100	100
500	0	0	0	0	0	0	0	100	100
1000	0	0	0	0	0	0	0	100	100
5000	0.28	0	30.6	13.3	0	0	0	100	100

*: KLK is a peptide whose amino acid sequence is KLKLLLLLKLK-NH_2.

As can be seen in Table 12 above, it was clearly determined that the peptides of the invention do not cause cell lysis, which was a surprising finding to guarantee the safety of the antibiotic peptides of the invention, since both of controls including a commercially available antibiotic such as Amphotericin B, cause cell lysis which is one of serious untoward effects in a human body administered with the antibiotics.

As clearly illustrated and demonstrated above, the present invention provides novel antibiotic peptides which possess superior antibacterial and/or antifungal activities causing no untoward effects such as cell lysis, and to pharmaceutical compositions containing said peptides as active ingredients.

WHAT IS CLAIMED IS:

1. Acid- or amide-form peptides which possess both antibacterial and antifungal activities, which are represented by the general formula(I), and analogues thereofs including enantiomers, retro-inversoes and derivatives where at most 3 neighboring amino acids located in each of N- and/or C-terminals, or at most 2 neighboring amino acids in the mid-part of the peptides, are substituted with D-form amino acids, respectively:

$$\alpha^1\ \alpha^2\ \alpha^3\ \alpha^4\ \alpha^5\ V\ C\ V\ C\ \alpha^6 \qquad (I)$$

wherein,

α^1 is 2 to 4 residues of amino acid;
α^2 is N, K or V;
α^3 is vacant, or G, P, L or K;
α^4 is vacant or K;
α^5 is vacant or R; and,
α^6 is R, L or D.

2. The peptides of claim 1, wherein α^1 is selected from the group consisting of YC, FC, WS, FYC, KYC, PYC, KFYC and FFYC.

3. The peptides of claim 1, wherein C(Cys) is replaced with aminoisobutyric acid.

4. Acid- or amide-form peptides which possess both antibacterial and antifungal activities, which are represented by the general formula(II), and analogues thereofs including enantiomers, retro-inversoes and derivatives where at most 3 neighboring amino acids located in each of N- and/or C-terminals, or at most 2 neighboring amino acids in the mid-part of the peptides, are substituted with D-form amino acids, respectively:

$\beta^1\ \beta^2\ \beta^3\ \beta^4\ \beta^5\ \beta^6\ \beta^7\ \beta^8\ \beta^9\ \beta^{10}$ (II)

wherein,

β^1 is vacant, or 1 or 2 basic amino acids;

β^2 is vacant, or 1 or 2 hydrophobic or basic amino acids(provided that β^1 is vacant and β^2 is 1 residue of amino acid, Pro and Tyr are excluded);

β^3 is 2 amino acids selected from the group consisting of hydrophobic amino acids and Cys;

β^4 is 1 or 2 amino acids(provided that β^4 is 1 residue of amino acid, Pro and acidic amino acids are excluded; and, provided that β^4 is 2 amino acids, both of which should not be acidic amino acids);

β^5 is 1 or 2 basic amino acids;

β^6 is vacant, or a hydrophobic amino acid;

β^7 is an amino acid selected from the group consisting of hydrophobic aromatic and aliphatic amino acids, Cys and Ser;

β^8 is a hydrophobic amino acid;

β^9 is an amino acid selected from the group consisting of hydrophobic aromatic and aliphatic amino acids, Cys and Ser (provided that β^7 is a hydrophobic aliphatic amino acid or Ser, β^9 should be a hydrophobic aromatic amino acid or Cys); and,

β^{10} is 1 or 2 basic amino acids.

5. Acid- or amide-form peptides which possess both antibacterial and antifungal activities, which are represented by the general formula(III), and analogues thereofs including enantiomers, retro-inversoes and

derivatives where at most 3 neighboring amino acids located in each of N- and/or C-terminals, or at most 2 neighboring amino acids in the mid-part of the peptides, are substituted with D-form amino acids, respectively:

$$\gamma^1\ \gamma^2\ \gamma^3\ \gamma^4\ \gamma^5 \qquad \text{(III)}$$

wherein,

γ^1 is 1 to 4 residues of amino acid;
γ^2 is 2 to 4 hydrophobic amino acids;
γ^3 is 1 or 2 basic amino acids;
γ^4 is 2 to 4 hydrophobic amino acids; and,
γ^5 is 1 to 3 amino acids containing at least one of basic amino acids(provided that γ^5 is more than 2 amino acids, basic amino acids are directed to N-terminal).

6. Acid- or amide-form peptides which possess antifungal activity, which are represented by the general formula(IV), and analogues thereofs including enantiomers, retro-inversoes and derivatives where at most 3 neighboring amino acids located in each of N- and/or C-terminals, or at most 2 neighboring amino acids in the mid-part of the peptides, are substituted with D-form amino acids, respectively:

$$\delta^1\ (ab)_{n1}\ (ba)_{n2}\ c\ \delta^2 \qquad \text{(IV)}$$

wherein,

δ^1 is 1 to 4 residues of amino acid;
a is a hydrophobic aromatic amino acid;
b is a hydrophobic aliphatic amino acid;
n1 is an integer of 1 or 2;
n2 is an integer of 1, 2 or 3(provided that

n1 is 1, n2 is 2 or 3; and, provided that n1 is 2, n2 is 1 or 2);

c is vacant, or a hydrophobic amino acid; and,

δ^2 is 1 or 2 basic amino acids.

7. A pharmaceutical composition which shows both antibacterial and antifungal activities, which contains at least one of peptides in claim 1 as active ingredient and pharmaceutically acceptable carriers.

8. A pharmaceutical composition which shows both antibacterial and antifungal activities, which contains at least one of peptides in claim 4 as active ingredient and pharmaceutically acceptable carriers.

9. A pharmaceutical composition which shows both antibacterial and antifungal activities, which contains at least one of peptides in claim 5 as active ingredient and pharmaceutically acceptable carriers.

10. A pharmaceutical composition which shows antifungal activity, which contains at least one of peptides in claim 6 as active ingredient and pharmaceutically acceptable carriers.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 96/00034

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 07 K 7/04; A 61 K 38/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 07 K 7/04, 7/06, 7/08; A 61 K 38/04, 38/08

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	The Journal of Biochemistry, Vol.16, No.1, July 1994, (TOKYO), H.J.MOON et al. "Purification and Molecular Cloning of cDNA for an Inducible Antibacterial Protein from Larvae of the Coleopteran, Tenebrio molitor" pages 53-58; totality.	1,7
P,A	Chemical Abstracts, Vol.123, No.25, 18 December 1995 (Columbus, Ohio, USA), page 447, right column, abstract No.333052k, Y.H.JUNG et al.:"Biochemical and molecular characterization of an antifungal protein from Tenebrio molitor larvae", & Mol.Cells 1995, 5(3), 287-92.	1
P,A	EP 0 665 239 A1 (CONSIGLIO NAZIONALE DELLE RICERCHE) 02 August 1995 (02.08.95), claims 1-5.	1

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 May 1996 (24.05.96)	05 June 1996 (05.06.96)

Name and mailing address of the ISA/ AT	Authorized officer
AUSTRIAN PATENT OFFICE Kohlmarkt 8-10 A-1014 Vienna	Wolf
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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 96/00034

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Claims 1-3 and 7; claims 4 and 8; claims 5 and 9; claims 6 and 10 relate to peptides with antibacterial and antifungal activities and pharmaceuticals containing these peptides. However, each of said groups of peptides has an other sequence of amino acids and differ to each other with respect of their composition.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-3 and 7

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR 96/00034

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
EP A1 665239	02-08-95	keine - none - rien	